Liraglutide protects injured neurons through down-regulating RAGE expression in ischemic rat brain after MCAO

Pengtian Lu1, Yuejia Song2, Jiyuan Zhu1, Hongmei Meng1, Na Ye1, Mengdi Wang1, Qi Liu1, Zhen Zhang1, Yuxin Pang1, Jiping Qi1, He Wu1
Departments of 1Pathology, 2Endocrinology, The First Affiliated Hospital of Harbin Medical University, Harbin, P. R. China

Abstract: Cerebral ischemia is a severe disease. The number of patients with cerebral ischemia has been on the rise over the past 20 years. Receptor of advanced glycation end products (RAGE), a member of the immunoglobulin family, was increased after cerebral ischemia and had effects on the brain. Liraglutide, a GLP-1 analogue, was able to cross the blood brain barrier and exhibit neuroprotective effects. The aim of this study was to investigate whether liraglutide exhibited its neuroprotective effect through down-regulating RAGE expression in ischemic rat brain after MCAO. Sprague-Dawley (SD) rats were treated with liraglutide or saline intraperitoneally after MCAO. Rats were assessed for neurological deficit and then sacrificed 24 h after occlusion. The results revealed that liraglutide could reduce the neurologic score and neuron injury. Furthermore, with decreased RAGE expression, microvessel density (MVD) was increased. In addition, the level of vascular endothelial growth factor (VEGF) expression was suppressed in rat ischemic brain at a lower concentration of liraglutide, while the level of VEGF expression was elevated at a higher concentration of liraglutide. Taken together, the results of the study indicated that liraglutide could protect the ischemic brain through down-regulating RAGE expression and increasing microvessel density.

Keywords: Cerebral ischemia, liraglutide, RAGE, neuron injury, VEGF, MVD

Introduction
Cerebral ischemia is a severe disease with high mortality [1]. The number of patients with cerebral ischemia has increased over the last 20 years [2]. Patients suffering from cerebral ischemia are left with moderate or severe disability 3 months later without prompt and efficacious treatment [3]. After cerebral ischemia, neurologic dysfunction occurs following neuronal injury and death [1]. Currently, the FDA-approved pharmacological treatment for cerebral ischemic is to use recombinant tissue plasminogen activator (rt-PA) to dissolve the clot and restore the blood supply in the ischemic brain. However, this carries the risk of cerebral hemorrhage and has no neuron protective effect [4]. Thus, new therapeutic strategies to protect injured neurons after cerebral ischemia need to be found.

Receptor of advanced glycation end products (RAGE), a member of the immunoglobulin family, is expressed in neurons in the brain [5]. RAGE is also a pattern recognition receptor that can bind multiple ligands, such as AGEs (advanced glycation end products), S100, and HMGB-1 (high mobility group box-1) [6]. Previous studies indicated that RAGE was involved in various inflammatory mechanisms, including oxidative stress, and participated in the occurrence and progress of some diseases such as Alzheimer Disease (AD) and diabetes mellitus (DM) by binding its ligands [7, 8]. Zimmerman et al. revealed that RAGE was increased in the brain after cerebral ischemia, and activated RAGE could exhibit neurotoxicity in the ischemic brain [9]. Further studies demonstrated that RAGE was expressed in neurons [10] and activation of RAGE in neurons could produce oxidative stress and induce the death of neurons [11, 12]. According to Lee's work, RAGE...
Liraglutide protects injured neurons

was elevated after cerebral ischemia, and activated RAGE was able to stimulate reactive oxygen species that could induce the death of neurons [5].

Glucagon-like peptide-1 (GLP-1), an endogenous incretin hormone of 30-amino acids, is produced by intestinal L cells. GLP-1 has an affinity for the GLP-1 receptor (GLP-1R) [13]. Previous studies demonstrated that GLP-1 had the ability to reduce oxidative stress in peripheral sensory neuropathy [14]. In the brain, GLP-1R was mainly expressed in the hypothalamus, the hippocampus and neurons and played a neuroprotective role in the brain [15]. Natural GLP-1 is easily degraded by dipeptidyl peptidase-IV (DPP-4), and it could not exhibit its therapeutic effect due to its short half-time [16].

Liraglutide, a GLP-1 analogue, is able to cross the blood brain barrier [17] and provide treatment for DM patients [18]. In recent years, some studies revealed that liraglutide could treat AD patients and ameliorate cognitive function in AD mice [15]. Sato et al. reported that liraglutide could exert its neuroprotective effect through ameliorating the function of neurons and reducing oxidative stress after transient cerebral ischemia and increasing the expression of vascular endothelial growth factor (VEGF) [19].

Liraglutide is a neuroprotective factor. However, the mechanism underlying its neuroprotective effect is still unclear. Thus, the aim of the present study is to demonstrate that liraglutide plays its neuroprotective role through down-regulating the expression of RAGE after MCAO. The study also reveals that liraglutide may be involved in angiogenesis in the ischemic rat brain.

Materials and methods

Subjects

Adult male SD rats (200-250 g) were obtained from the Experimental Animal Center, the second affiliated hospital of Harbin Medical University, Heilongjiang Province, China. Rats were housed in a conventional standard cage under appropriate temperature and humidity control with a 12 h light/12 h dark cycle and with access to food and water throughout the experiment. All experimental operations were approved by the Institutional Animal Care and Use Committee (IACUC) at Harbin Medical University.

Animals and MCAO

Rats (n=60) were randomly divided into three groups: (1) control group (n=12), (2) MCAO group (n=12), and (3) liraglutide (Novo Nordisk Pharma Ltd., Gladsaxe, Denmark)-treated group (n=36), including 3 sub-groups: 0.3 mg/kg liraglutide group (n=12), 0.5 mg/kg liraglutide group (n=12), and 0.7 mg/kg liraglutide group (n=12). Liraglutide was injected intraperitoneally after MCAO. The dose and administration of liraglutide in this study was approved by previous studies [19] and our preliminary study.

Rats were anesthetized, and body temperature was maintained at 37°C. A line model (diameter =0.234 mm) was inserted into the internal carotid artery to block the middle cerebral artery as previously described [20]. All rats were assessed for neurological deficit and then sacrificed 24 h after MCAO. Brain tissue was processed for western blot analysis and immunohistochemical staining.

Neurologic evaluation

The neurologic evaluation was conducted using a scoring standard described by Enrique et al., 1989 [20]. The neurology evaluations were scored on a five-point scale: a score of 0 represented no neurologic deficit, a score of 1 (failure to extend left fore paw) indicated a mild focal neurologic deficit, a score of 2 (circling to the left) meant a moderate focal neurologic deficit, and a score of 3 (falling to the left) indicated a severe focal deficit; rats with a score of 4 did not walk spontaneously and had a depressed level of consciousness.

FJB staining

Fluoro-Jade B (FJB) has high sensitivity and specificity polyanionic fluoresce that can bind degenerated neurons. FJB staining of brain slides was conducted as previously described with some modifications [21]. Slides were then subjected to the following procedures. Briefly, selected sections were first incubated in a solution of 1% NaOH in 80% ethanol for two minutes. Second, the sections were placed into 70% ethanol for two minutes. Third, slides were washed with distilled water for two minutes.
Liraglutide protects injured neurons

Samples were electrophoresed using SDS–PAGE at 80 V for 2 h and transferred to PVDF membranes (Millipore, Bedford, MA, USA) at 300 mA for 1.5 h. The membranes were blocked with 0.1% BSA (735094, Beyotime Biotech, Shanghai, China) for one hour at room temperature and then incubated with rabbit anti-RAGE (1:200; cat. on. ab65965; Abcam, Cambridge, MA, USA), mouse anti-VEGF (1:200; sc7269; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and mouse anti-β-actin (1:1,000, TA-09; Zhongshan Biotechnology Co., Ltd., Beijing, China) overnight at 4°C. After washing with T-BST, the membranes were incubated with goat anti-mouse or anti-rabbit secondary antibody (1:500, cat. no. ZB-2305 and ZB-2301, Zhongshan Biotechnology Co., Ltd., Beijing, China) for 1 h at 37°C. Protein bands were visualized with BeyoECL Plus kits (P0018, Beyotime Biotech, Shanghai, China). The optical densities of the detected proteins were obtained using a UVP Software iBox 500 Imaging System (Upland, CA, USA).

Statistical analysis

The data were expressed as the mean ± SD. Statistical analysis was performed by one-way ANOVA using SPSS 17.0. LSD post hoc tests were used for between groups comparison. Statistical significance was set at P<0.05.

Results

Liraglutide ameliorated the neurologic score

To determine whether liraglutide affected neurologic function, neurologic score of control, MCAO and liraglutide-treated rats were evaluated 24 h after MCAO. The results indicated that the neurologic score of MCAO rats was higher than that of the control group, while the neurologic score of liraglutide-treated rats was lower than that of MCAO rats. Moreover, liraglutide could ameliorate the neurologic score in a dose-dependent manner. Data were mean ± SD. *P<0.05 vs MCAO group.

Liraglutide reduced the number of injured neurons in the rat brain after cerebral ischemia

To ascertain the effect of liraglutide treatment on degenerating neurons, Fluoro-Jade B (FJB) staining of control, MCAO, and liraglutide-treated rats was carried out, as shown in Figure 2.
Liraglutide protects injured neurons

The results indicated that the number of degenerated neurons in the ischemic penumbra of MCAO rat brain was higher than that of the control group, while the number of degenerated neurons in the ischemic penumbra of liraglutide-treated rats was significantly reduced at 24 h after MCAO, compared with that of MCAO rats. The results suggested that at concentrations of 0.5 mg/kg and 0.7 mg/kg, liraglutide could markedly decrease the number of degenerated neurons (P<0.05) (Figure 2).

**Liraglutide decreased the expression of RAGE in the rat brain after cerebral ischemia**

To examine whether liraglutide could reduce the expression of RAGE, immunohistochemical labeling (Figure 3A) and western blot analysis (Figure 3B) were used in the present study. In the immunohistochemical staining (Figure 3A), RAGE was expressed on the cell membrane. RAGE positive cells were increased in the ischemic penumbra of MCAO rat brains compared with the control group. However, 24 h after MCAO, RAGE positive cells in liraglutide-treated groups were significantly reduced compared with the MCAO group. At concentrations of 0.5 mg/kg and 0.7 mg/kg, liraglutide exhibited significant reduction of injured neurons (P<0.05). Moreover, the immunoreactivity of the RAGE positive band (48 kDa) was similar to the results of immunohistochemistry, and the intensity of the band in the liraglutide-treated rats was weaker than in MCAO rats. RAGE expression was significantly reduced in all three liraglutide-treated groups (P<0.05).

**Liraglutide increased microvessel density in the rat brain after cerebral ischemia**

To examine whether liraglutide could increase the microvessel density in the cerebral ischemic penumbra 24 h after MCAO, CD34 immunohistochemical staining of control, MCAO, and liraglutide-treated rats was conducted, as shown in Figure 4. The microvessel density in the ischemic penumbra of MCAO rat brain was elevated compared with that of control rats, while the microvessel density of liraglutide-treated rats was higher than that of MCAO rats. Moreover, the results also suggested that liraglutide could increase the microvessel density in a dose-dependent manner. At concentrations of 0.3 mg/kg, 0.5 mg/kg, and 0.7 mg/kg, lira-
Liraglutide protects injured neurons

However, at a concentration of 0.7 mg/kg, liraglutide significantly increased VEGF expression in ischemic brains compared with the MCAO rats (P<0.05). The immunoreactivity of the VEGF positive band (21 kDa) was similar to the results of immunohistochemistry. The results of western blot analysis demonstrated that liraglutide significantly reduced the expression of VEGF at concentrations of 0.3 mg/kg and 0.5 mg/kg (P<0.05), but at 0.7 mg/kg, liraglutide significantly increased the level of VEGF (P<0.05).

Discussion

The aim of this study was to assess whether liraglutide has a protective effect on rat brains.
Liraglutide protects injured neurons

![Image](https://example.com/image.png)

**Figure 4.** Liraglutide increased the Microvessel Density. The CD34 immunohistochemical staining suggested that (A) Micro vessel density (MVD) in the control group was small. (B) MVD was increased after MCAO. (C) MVD was higher in Liraglutide (0.3 mg/kg)-treated group (D) Liraglutide (0.5 mg/kg)-treated group (E) Liraglutide (0.7 mg/kg)-treated group than MCAO group. (F) The graph showed that there is significantly increased between liraglutide-treated groups and MCAO group. Data were mean ± SD. *P<0.05 vs MCAO group.

After MCAO. Neurologic evaluation, FJB staining, immunohistochemical staining and western blots were used to measure the neurologic deficit, number of injured neurons, microvessel density, and the expression of RAGE and VEGF in MCAO rats receiving liraglutide.

Neurons in the brain are injured due to interruption of the blood supply after cerebral ischemia. Briyal et al. suggested that oxidative stress, apoptosis and inflammation occur after cerebral ischemia [22]. These factors cause damage or the death of neurons. Neurons are permanent cells, and once they are destroyed, the damage cannot be reversed.

24 h after cerebral ischemia, there was a severe neurologic deficit, as well as an increase in injured neurons and RAGE expression. Liraglutide treatment resulted in fewer neurologic deficits and a lower number of injured neurons. These beneficial results were associated with inhibition of RAGE. Liraglutide also increased angiogenesis in cerebral ischemia. These results suggested that liraglutide may attenuate cerebral injury in ischemic rats.

Liraglutide, a GLP-1 analogue, shares 97% amino-acid sequence identify with GLP-1, and is a new drug that can be used for treating DM. Recently, studies demonstrated that liraglutide could ameliorate cognitive function and be used for treating AD [15]. Both GLP-1 and its analogue, liraglutide, have multiple physiologic functions, such as inhibition of glucagon secretion, stimulation of cell division, inhibition of reactive oxygen species (ROS) release and anti-apoptotic properties. In the central nervous system, both GLP-1 and liraglutide can across the blood brain barrier readily and access the cerebral parenchyma directly [23]. GLP-1 receptor is distributed in various tissues, including the brain in rats and humans, and is widely expressed throughout the brain [24]. For the first time, Hou et al. showed that in a mouse model of intracerebral hemorrhage, liraglutide could suppress brain edema and neuroinflammation [25]. Furthermore, Briyal et al. demonstrated that pretreatment with liraglutide played a neuroprotective role in cerebral ischemia. Liraglutide could reduce the infarct volume, oxidative stress and apoptosis [22]. In our study, rats received a middle cerebral artery occlusion, resulting in severe neurological deficits with paralyzed limbs. Liraglutide was able to reduce neurological deficits compared with the MCAO group. The results of neurological...
Liraglutide protects injured neurons

Fang et al. indicated that RAGE expression was relatively low in most tissues, including the central nervous system (CNS), in mature healthy animals, whereas during disease states such as AD, DM and brain injury, RAGE would increase [27]. Previous studies demonstrated that activation of RAGE signaling could damage brain tissues [28], and RAGE acted as a neurotoxicity factor in Alzheimer’s disease (AD) [6]. Furthermore, Dorecka et al. reported that GLP-1 played a key role in protecting human retinal pigment epithelial cells through reducing RAGE expression [30]. In the present study, the level of RAGE was measured to evaluate the degree of brain injury. Our results revealed that cerebral ischemia increased the level of RAGE and that lira-

Cerebral ischemia is a severe disease [1], and patients who suffer a cerebral ischemia are left with a moderate or severe disability [3]. Recently, studies focused on the basic mechanisms of cerebral ischemia suggested that oxidative stress represented a main element in the occurrence and development of cerebral ischemia [22]. RAGE, receptor of advanced glycation end products, is a pattern recognition receptor that can bind multiple ligands. Activation of RAGE could trigger several signaling pathways, which could lead to pathologic dam-

Figure 5. The effect of Liraglutide on VEGF (A). Immunohistochemical staining indicated that (a) VEGF expression in the control group. (b) VEGF expression was increased after MCAO. (c) VEGF expression in liraglutide (0.3 mg/kg)-treated group. In this group, VEGF expression was lower compared with MCAO group. (d) VEGF expression in liraglutide (0.5 mg/kg)-treated group. VEGF expression was higher compared with MCAO group. (e) VEGF expression was lower compared with liraglutide (0.7 mg/kg)-treated group. (e) Expression of VEGF in liraglutide (0.7 mg/kg)-treated group, the expression of VEGF was significantly higher than other groups. (f) The graph demonstrated that there is significantly increased in liraglutide (0.7 mg/kg)-treated groups compared with MCAO group. Data were mean ± SD. *P<0.05 vs MCAO group. The expression of VEGF protein analysis with β-actin in Western Blot (B). VEGF protein level along with β-actin in control group, MCAO group and liraglutide (0.3, 0.5, 0.7 mg/kg)-treated groups. Data were mean ± SD. *P<0.05.

age, such as oxidative stress, inflammation and apoptosis [6-8]. RAGE activation elicits a loop which could produce more oxidative stress and subsequently evoke cell damage [26]. Fang et al. indicated that RAGE expression was relatively low in most tissues, including the central nervous system (CNS), in mature healthy animals, whereas during disease states such as AD, DM and brain injury, RAGE would increase [27].
Liraglutide protects injured neurons

Liraglutide treatment after cerebral ischemia could reduce the expression of RAGE in the brains of MCAO rats. These results demonstrated that the GLP-1 analogue, liraglutide, played a paramount role as a neuroprotector against cerebral ischemic injury through down-regulating RAGE expression. Furthermore, liraglutide was able to reduce RAGE expression in a dose-dependent manner.

Evidence showed that the natural recovery process in infarct regions after cerebral ischemia is angiogenesis [29]. Aronis suggested that GLP-1 could promote angiogenesis through the Akt, Src, and PKC pathways in human umbilical vein endothelial cells (HUVECs) [31]. Moreover, Kang et al. demonstrated that GLP-1 against Ex-4 has angiogenic effects in vitro and in vivo [32]. Recently, a few studies indicated that treatment with liraglutide both before and after cerebral ischemia could reduce the infarct volume [19, 22]. This may be the effect of GLP-1 on angiogenesis. Thus, in our study, we used CD34 as a biomarker of microvessels to track angiogenesis [33]. The results showed that treatment with liraglutide after cerebral ischemia significantly increased the expression of CD34 positive cells, compared with the MCAO group. Furthermore, liraglutide was able to increase the CD34 positive cells in a dose-dependent manner. The results suggested that liraglutide may protect the brain against ischemic injury through angiogenesis.

In addition, we would like to know whether GLP-1 could stimulate angiogenesis through upregulating VEGF. The Ding study reported that VEGF could stimulate angiogenesis through the VEGF/VEGFR-Src-ERK pathway [34].

The Sun’s study showed that GLP-1 could stimulate angiogenesis through the GLP-1R/Akt/VEGF pathway in a murine model of myocardial infarction [35]. Additionally, Kang reported that exendin-4, another GLP-1 analog, could increase the level of VEGF in a model of ischemic hindlimbs at the dosage of 1 mg/kg [32]. Sato et al. demonstrated that liraglutide, another GLP-1 analog, could increase the expression of VEGF in a model of cerebral ischemia at the dosage of 0.7 mg/kg [19]. In our study, the result indicated that liraglutide at both the 0.3 mg/kg and 0.5 mg/kg concentrations could not increase the level of VEGF after cerebral ischemia. In contrast, it reduced VEGF expression. However, at the dosage of 0.7 mg/kg, liraglutide could increase the level of VEGF, coinciding with the results of the Sato study. The main mechanism of this phenomenon is unknown and remains to be discovered. Possible explanations include that low dose GLP-1 does not have enough energy to bind to the GLP-1 receptor or that low dose GLP-1 fails to activate receptors other than the GLP-1 receptor, which in turn may activate anti-VEGF signaling pathways to decrease the expression of VEGF. Meanwhile, a higher dose of GLP-1 could have enough energy to bind the GLP-1 receptor and then upregulate the expression of VEGF. Further investigations are required to identify the mechanisms by which liraglutide regulates angiogenesis in infarct zones of the ischemic brain.

In summary, the results indicated that liraglutide could protect neurons through down-regulating the expression of RAGE and promoting angiogenesis in the ischemic brain. Liraglutide may be a candidate therapeutic drug to treat patients with cerebral ischemia.

Acknowledgements

This work was supported by the Graduate Innovation Foundation of Harbin Medical University (YJSCX2014-27HYD).

Disclosure of conflict of interest

None.

Address correspondence to: Drs. Jiping Qi and He Wu, Department of Pathology, The First Affiliated Hospital of Harbin Medical University, 23 Youzheng Street, Nangang District, Harbin 150000, P. R. China. Tel: +86-451-85555809; Fax: +86-451-85555809; E-mail: qijiping2003@163.com (JPQ); wuhe_2008@hotmail.com (HW)

References


Liraglutide protects injured neurons


[27] Fang F, Lue LF, Yan S, Xu H, Luddy JS, Chen D, Walker DG, Stern DM, Yan S, Schmidt AM, Chen JX and Yan SS. Rage-dependent signal-
Liraglutide protects injured neurons


[34] Ding Q, Tian XG, Li Y, Wang QZ and Zhang CQ. Carvedilol may attenuate liver cirrhosis by inhibiting angiogenesis through the VEGF-Src-ERK signaling pathway. World J Gastroenterol 2015; 21: 9566-9576.